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The Transcriptional Control of Trunk Neural Crest Induction, Survival, and Delamination

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Summary

Trunk neural crest cells are generated at the border between the neural plate and nonneural ectoderm, where they initiate a distinct program of gene expression, undergo an epithelial-mesenchymal transition (EMT), and delaminate from the neuroepithelium. Here, we provide evidence that members of three families of transcription induce these properties in premigratory neural crest cells. Sox9 acts to provide the competence for neural crest cells to undergo an EMT and is required for trunk neural crest survival. In the absence of Sox9, cells apoptose prior to or shortly after delamination. Slug/Snail, in the presence of Sox9, is sufficient to induce an EMT in neural epithelial cells, while FoxD3 regulates the expression of cell-cell adhesion molecules required for neural crest migration. Together, the data suggest a model in which a combination of transcription factors regulates the acquisition of the diverse properties of neural crest cells.

Introduction

Neural crest cells (NCCs) are generated at the border between the neural plate and nonneural ectoderm. These cells initiate a distinct program of gene expression; undergo an epithelial-mesenchymal transition; delaminate from the neuroepithelium; and migrate into the periphery, where they differentiate into a diverse set of derivatives, including peripheral nervous system neurons and glia and pigment cells (Le Douarin and Kalcheim, 1999). Induction of NCCs appears to depend on signals emanating from nonneural ectoderm or nonaxial mesoderm, and progress has been made in identifying and characterizing these signals (reviewed in Knecht and Bronner-Fraser, 2002). Within responding cells, these signals are thought to initiate a program that modifies the transcriptional activity, cell adhesion, cytoskeleton assembly, and cell-matrix interaction of prospective NCCs (reviewed in Savagner, 2001). These changes establish NCC identity and promote an epithelial-mesenchymal transition (EMT) involving the reorganization of the actin cytoskeleton, loss of epithelial polarity, and alterations in cell-cell adhesive properties (Duband et al., 1995; Knecht and Bronner-Fraser, 2002). The induction of NCC identity and the initiation of an EMT appear to be separable (Newgreen and Minichiello, 1995; Sela-Donenfield and Kalcheim, 1999); although, during normal neural crest development, these events occur in a spatially and temporally coordinated manner.

Despite the identification of a number of genes activated in prospective NCCs, the steps at which these genes act and how the acquisition of the different features of NCCs are integrated remain poorly defined. Sox9, a member of the SoxE subgroup of HMG-containing transcription factors, and the winged-helix transcription factor FoxD3 are sufficient to induce a number of the properties of NCCs in chick neural tube cells (Cheung and Briscoe, 2003; Dottori et al., 2001; Kos et al., 2001). Further supporting a role for Sox9 in NCC induction, loss-of-function analyses in *Xenopus* suggest that Sox9 is required for cranial neural crest formation (Spokony et al., 2002). The heterozygous lethality of Sox9 null mice has hampered experiments to address the requirement for Sox9 in NCC induction in amniotes (Bi et al., 2001). Moreover, consistent with the idea that the specification and EMT of NCCs are independently controlled, and although Sox9 and FoxD3 are sufficient to induce many characteristics of NCC differentiation, neither efficiently promotes an EMT (Cheung and Briscoe, 2003; Dottori et al., 2001). Candidates for orchestrating an EMT include the small G protein RhoB (Liu and Jessell, 1998) and the zinc finger transcription factors Slug and Snail (Bolos et al., 2003; Cano et al., 2000). RhoB is expressed in premigratory and early migrating NCCs, and inhibition of Rho activity prevents neural crest delamination (Liu and Jessell, 1998). Overexpression of members of the Snail family increases the number of migratory NCCs in cranial regions of chick embryos (del Barrio and Nieto, 2002), and inhibition of Slug function reduces NCC migration in *Xenopus* and chick (LaBonne and Bronner-Fraser, 2000; Nieto et al., 1994).

In this study, we dissect the cell intrinsic pathway of NCC induction in amniotes and examine the hierarchical relationship between the genes involved. Analysis of mouse embryos lacking Sox9 indicates that, in the absence of Sox9, trunk neural crest specification is initiated, but NCCs apoptose shortly afterward. Conversely, gain-of-function experiments reveal that specification of trunk NCCs involves the coordinated activity of Sox9, FoxD3, and Slug. Each transcription factor appears to regulate the acquisition of distinct NCC properties, while the combined expression of Sox9, Slug, and FoxD3 in neural cells appears to induce cells that manifest all the principal transcriptional and morphological characteristics of NCCs. Together, these findings indicate that NCC induction is dependent on an integrated cell intrinsic network of transcription factors

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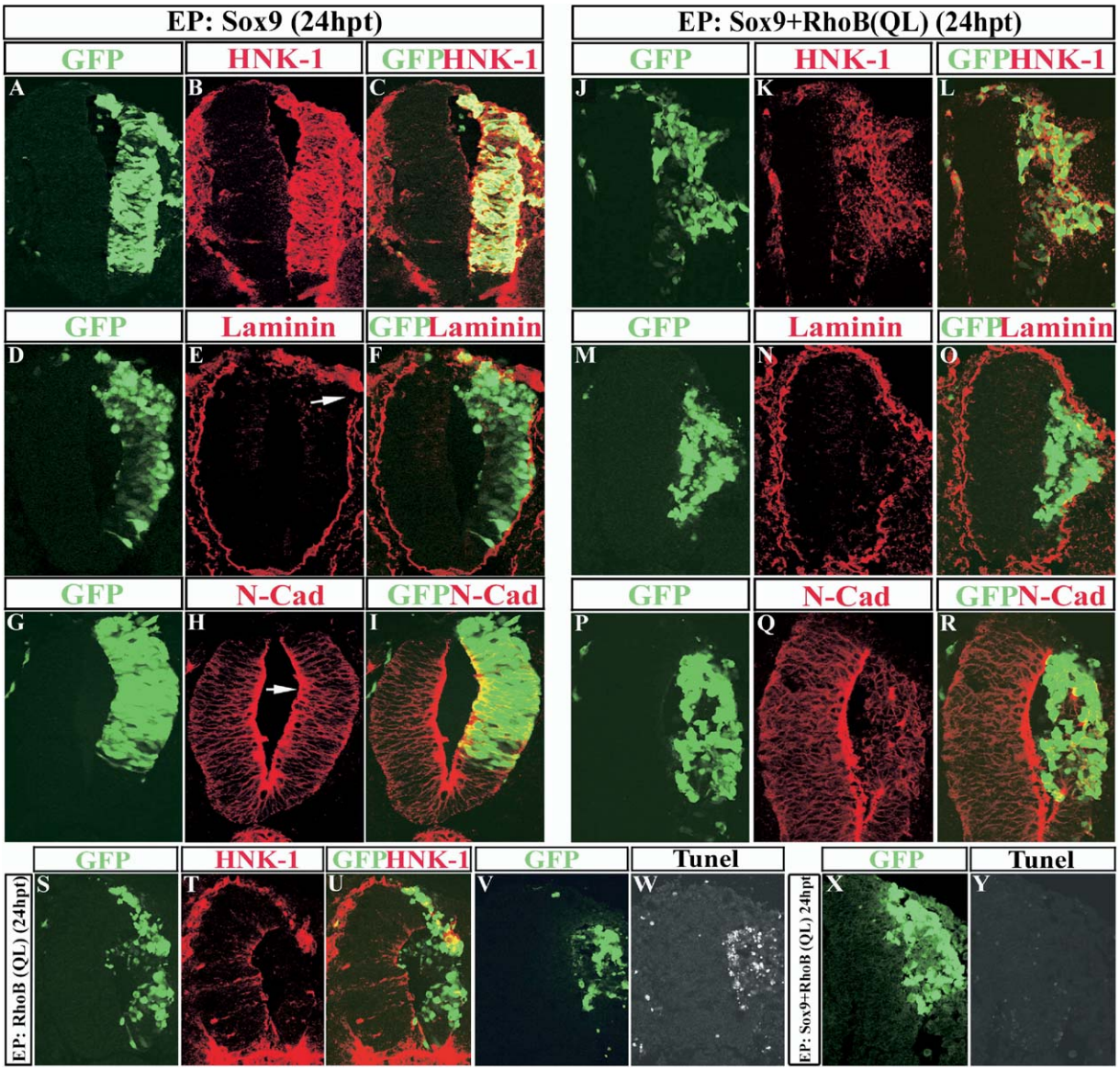

Figure 1. Coexpression of Sox9 and RhoB Induces Neural Crest Cell Differentiation and an Epithelial-Mesenchymal Transition

(A–I) In ovo electroporation (EP) of Sox9 assayed 24 hr posttransfection (hpt). (A–C) Forced expression of Sox9 induces HNK-1 expression. (D–F) Laminin expression indicates that most Sox9-transfected cells remain within the neural tube; a few cells were observed delaminating from the dorsal neural tube where Laminin expression was downregulated (arrow). (G–I) Sox9 expression does not affect N-Cad expression; the arrow indicates apical concentration of N-Cad.

(J–R) Cotransfection of Sox9 and constitutively active RhoB(QL) assayed 24 hpt. (J–L) Ectopic HNK-1 expression is induced in transfected cells, and these cells no longer appear to have a pseudostratified morphology. (M–O) Many transfected cells are observed delaminating from the neural tube, and Laminin expression is lost from the basal surface of the neural tube. (P–R) N-Cad expression continues in transfected cells, but the apical accumulation is lost.

(S–U) Forced expression of RhoB(QL) does not induce ectopic HNK-1 expression.

(V and W) A TUNEL assay indicates that RhoB-transfected cells are undergoing apoptosis.

(X and Y) Few apoptotic cells are detected in embryos cotransfected with Sox9 and RhoB(QL).

that control and coordinate the acquisition of trunk NCC properties.

Results

Coexpression of Sox9 and RhoB Induces NCC Differentiation and EMT Characteristics

Previous studies provided evidence that Sox9 was sufficient to induce a number of properties of neural crest cell differentiation; however, Sox9 did not efficiently promote an EMT or NCC delamination (Cheung and Briscoe, 2003). One candidate for promoting an EMT is the small GTPase, RhoB (Liu and Jessell, 1998). To investigate this possibility, we performed in ovo electroporation in the chick spinal cord at Hamburger and Hamiliton (HH) stage-10 to stage-12 chick embryos at a stage prior to NCC delamination. Misexpression of Sox9, alone, induced expression of the chick neural

crest marker HNK-1 in neuroepithelial cells (Cheung and Briscoe, 2003; Figures 1A–1C, n = 6), but transfected cells remained in a pseudostratified epithelium and only a few cells were observed delaminating ectopically from intermediate regions of the neural tube. Moreover, the basement membrane remained largely intact (Figures 1D–1F, n = 6). In addition, N-Cadherin (N-Cad), which is concentrated on the apical surface of neuroepithelial cells (Akitaya and Bronner-Fraser, 1992), was unaffected by the forced expression of Sox9 (Figures 1G–1I, n = 6). In contrast, coelectroporation of Sox9 and either a wild-type RhoB or dominant, active RhoB (QL; Mellor et al., 1998) resulted in dramatic changes in neuroepithelial cell morphology. Coexpression of Sox9 + RhoB(QL) induced ectopic HNK-1 expression; however, transfected cells no longer exhibited a pseudostratified appearance and instead acquired a more rounded shape and a loosely packed configuration characteristic of mesenchymal-like cells (Figures 1J–1L, n = 10). Many transfected cells were found migrating out of the neural tube, and there was a marked reduction in Laminin expression on the basal surface of the neural tube (Figures 1M–1O, n = 10). Moreover, transfected cells lacked the apical accumulation of N-Cad expression, and N-Cad was uniformly distributed around the cell surface (Figures 1P–1R, n = 10). Cotransfection of Sox9 and a version of RhoB mutated to lack signaling activity (Mellor et al., 1998) did not induce the characteristics of an EMT (not shown).

We next asked whether the expression of RhoB(QL) alone was able to induce any properties of NCC differentiation or an EMT. In contrast to cotransfection of Sox9 + RhoB(QL), when RhoB(QL) was electroporated alone, no ectopic HNK-1 expression was detected (Figures 1S–1U, n = 10). However, neural tube morphology was severely distorted on the transfected side of the embryo (Figures 1S–1U, n = 10). TUNEL assays showed extensive apoptosis in embryos transfected with RhoB(QL) (Figures 1V and 1W, n = 10), while embryos coelectroporated with Sox9 + RhoB(QL) contained few apoptotic cells (Figures 1X and 1Y, n = 12). These data suggest that the coexpression of Sox9 and RhoB(QL) is sufficient to initiate NCC differentiation and is characteristic of an EMT, while, in the absence of Sox9, RhoB activity is incompatible with the survival of neural cells.

Sox9 Is Required for the Generation of Trunk Neural Crest Derivatives

These findings raised the question of whether Sox9 is required for neural crest generation. To address this, we examined mouse embryos lacking Sox9. In mouse, as in chick, *Sox9* is transiently expressed in premigratory NCCs, and this population of cells also expresses the transcription factors *Sox10*, *FoxD3*, and *Snail* (Supplemental Figures S1A–S1P; see the Supplemental Data available with this article online). Due to the perinatal lethality of $Sox9^{null/+}$ heterozygous mice (Bi et al., 2001), we took advantage of a conditional null allele in mouse embryos (Akiyama et al., 2004).

We have focused our analysis on trunk neural crest generated between the forelimb and hindlimb level. First, we examined dorsal root ganglion (DRG) in Sox9 null embryos at E10.5, when ventrally migrating NCCs had begun to differentiate in the peripherery (Serbedzija et al., 1990). At E10.5, large numbers of neurons and glia, marked by Isl-1/2 and Sox10, respectively, were observed in the DRG of wild-type embryos (Figures 2C–2K and Supplemental Figures S2E, S2O, and S2S). In contrast, neuronal and glial generation in the periphery was significantly reduced caudal to the forelimb level in mutant embryos (Figures 2C–2K and Supplemental Figures S2F, S2H, S2P, and S2T). However, Isl-1/2 expression in the dorsal interneurons and motor neurons in the spinal cord appeared to be unaffected in Sox9 mutants (Figure 2G). Consistent with the reduction in expression of Isl-1/2 and Sox10 in the periphery of Sox9 mutants, the peripheral expression of other neuronal markers, *Brn3.0* and TuJ1 (Figures 2L and 2M and Supplemental Figures S2J, S2L, S2N, and S4R), and in glial markers, including *ErbB3*, *FoxD3*, and *Notch-1* (Figures 2N and 2O and Supplemental Figures S2A–S2D), was also markedly reduced. We next examined the formation of sympathetic ganglia characterized by the expression of markers that include Phox2b and Mash1 (Pattyn et al., 1999; Guillemot et al., 1993; Figures 2P–2S). In wild-type embryos, sympathetic ganglia can be identified coalescing adjacent to the dorsal aorta (Figures 2P and 2R). In contrast, in embryos lacking Sox9, expression of Phox2b and Mash1 in sympathetic ganglia was absent, and no evidence for ganglia formation could be found (Figures 2Q and 2S). However, Mash1 expression in the spinal cord was unaffected by the loss of Sox9 (Figure 2S). Although severely reduced in Sox9 null embryos, a small number of NCC derivatives continued to be generated at trunk levels (Figures 2C–2O); moreover, at rostral forelimb and cranial levels, NCCs were generated and were distributed in a manner similar to that of wild-type embryos (Supplemental Figures S2O–S2R). These results indicate that lack of Sox9 has less impact on the generation of anterior NCCs. Taken together, these findings indicate that Sox9 is required for the normal generation of trunk neural crest derivatives.

In Sox9 Mutants, NCCs Undergo Apoptosis after Induction

The reduction of trunk neural crest derivative formation in Sox9 null embryos prompted us to examine whether neural crest induction was initiated in Sox9 null embryos. The expression of roof plate and dorsal neural tube progenitor markers, including *Wnt1*, *GDF7*, *Lmx1a*, Pax7, Math1, Pax3, Msx1/2, and Zic2, was similar in wild-type and Sox9 null embryos (Supplemental Figure S4), as was the generation of dorsal neuronal subtypes identified by the expression of Isl-1/2, LH2A/B, and Lim1/2 (Figures 2F and 2G and Supplemental Figure S4S).

We next analyzed the expression of *FoxD3*, *Sox10*, *RhoB*, and *Snail*, markers characteristic of early neural crest development at E9.5, a time point at which NCCs had begun to emigrate from the premigratory neural crest region. At E9.5 and E10.5, the expression of *FoxD3* in premigratory and early emigrating NCCs was similar in wild-type embryos and Sox9 mutants (Figures 3A–3D and data not shown). Moreover, *Sox10* expression was maintained in the premigratory neural crest region and the early migratory NCCs in Sox9 mutant embryos (Figures 3E–3H). However, expression of *Sox10* along the NCC migration route in the periphery was re-

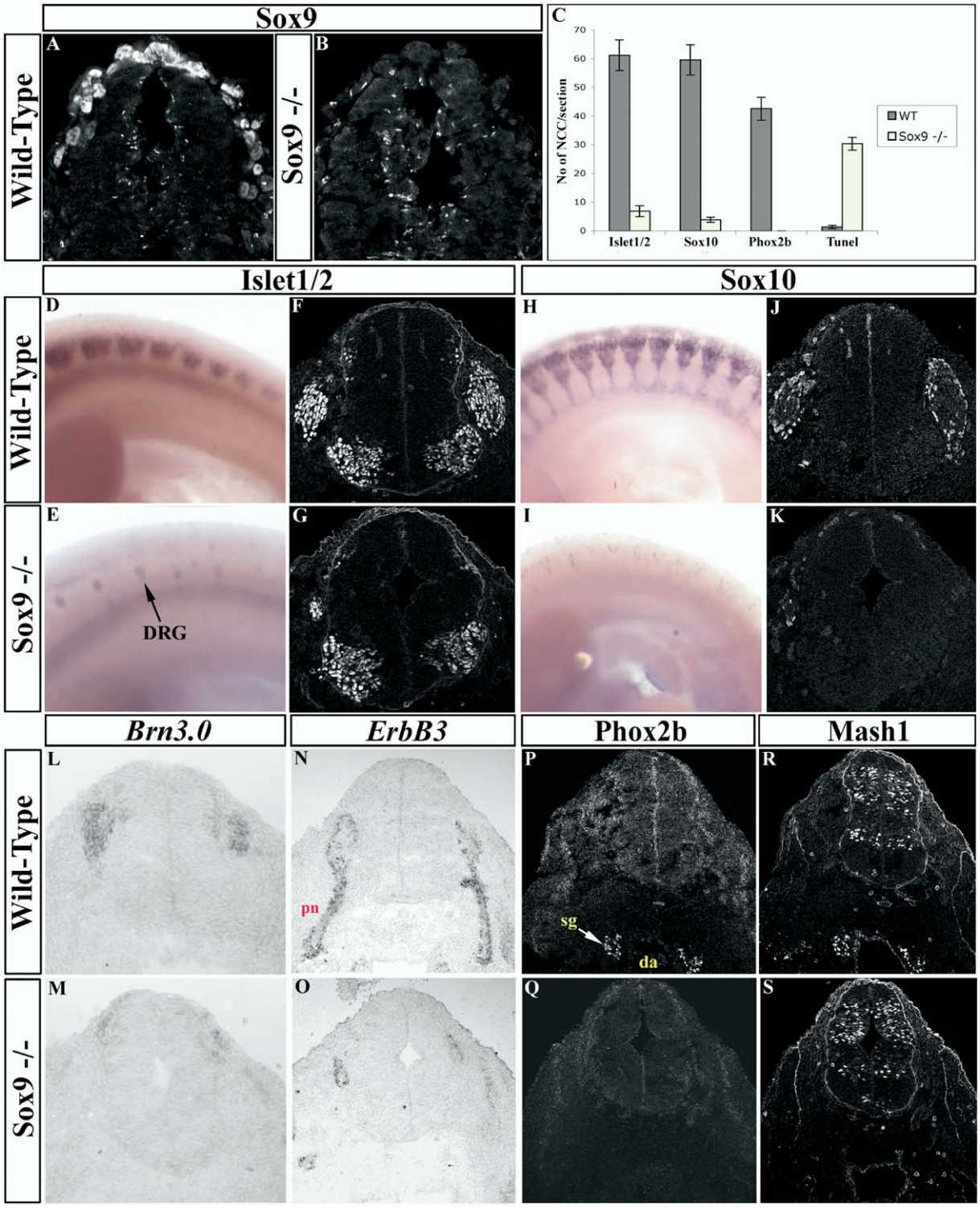

Figure 2. Sox9 Is Required for the Generation of Trunk Neural Crest Derivatives

(A and B) Immunofluorescence labeling with Sox9 on (A) wild-type and (B) Sox9$^{-/-}$ at E9.5.

(C) Quantification of neural crest cell expression of Isl-1/2, Sox10, Phox2b, and TUNEL (cells/section) on wild-type and Sox9$^{-/-}$ at E10.5 (mean ± SEM, n = 8 sections from at least four embryos).

(D–K) Whole-mount in situ hybridization on (D and H) wild-type and (E and I) Sox9$^{-/-}$ with (D and E) *Isl-1/2* and (H and I) *Sox10* showing significantly reduced expression of (E) *Isl-1/2* and (I) *Sox10* in the dorsal root ganglia (DRG) of Sox9$^{-/-}$ embryos. Immunofluorescence staining on sections from (F and J) wild-type and (G and K) Sox9$^{-/-}$ with (F and G) Isl-1/2 and (J and K) Sox10.

(L–O) In situ hybridization on (L and N) wild-type and (M and O) Sox9$^{-/-}$ with (L and M) *Brn3.0* and (N and O) *ErbB3*. Expression of *Brn3.0* in the (L) DRG and *ErbB3* expression in both DRG and peripheral nerve (pn) in the (N) wild-type; expression of *Brn3.0* and *ErbB3* in DRG was reduced in (M and O) Sox9$^{-/-}$. (O) Residual expression of *ErbB3* in peripheral nerve was observed in Sox9$^{-/-}$.

(P–S) Immunofluorescence labeling of sympathetic ganglia (sg) markers (P and Q) Phox2b and (R and S) Mash1 on (P and R) wild-type and (Q and S) Sox9$^{-/-}$ showing loss of expression in the sympathetic ganglia of (Q and S) Sox9$^{-/-}$. (S) Note that Mash1 was unaffected in the neural tube of Sox9$^{-/-}$. da, dorsal aorta.

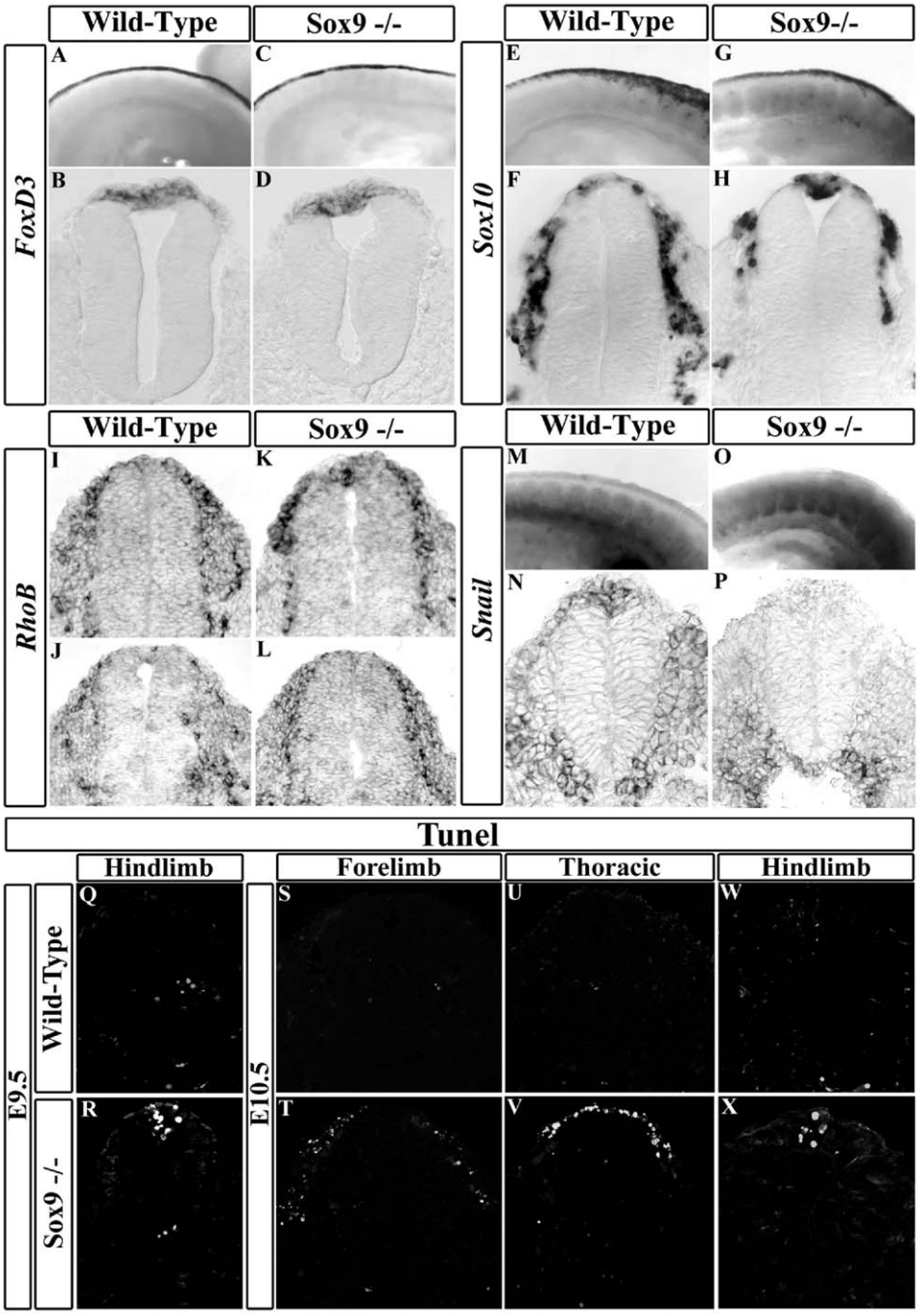

Figure 3. Neural Crest Cells Undergo Apoptosis in Sox9 Mutants

(A–P) Whole-mount in situ hybridization of wild-type and Sox9$^{-/-}$ embryos with (A and C) *FoxD3*, (E and G) *Sox10*, and (M and O) *Snail* at E9.5 and (B, D, F, H, N, and P) their corresponding sections at trunk level. Expression of (B and D) *FoxD3* and (F and H) *Sox10* was maintained in the emigrating neural crest cells both in the (B and F) wild-type and (D and H) Sox9$^{-/-}$, although the amount of *Sox10*$^{+}$ migrating neural crest cells was reduced in the mutants compared to the (F and H) wild-type. In situ hybridization on transverse sections of the (I and K) caudal and (J and L) trunk spinal cord at E10.5 with *RhoB* on (I and J) wild-type and (K and L) Sox9$^{-/-}$. (I) In the caudal neural tube, *RhoB* was mainly found in early migrating neural crest cells. (K) In Sox9 null embryos, *RhoB* was only detected in the premigratory and early migrating neural crest cells. (J) Anterior to the hindlimb level, *RhoB* was observed in the peripheral region of the DRG in wild-type embryos, whereas (L) *RhoB* expression was restricted to early migrating neural crest cells in Sox9$^{-/-}$ mutants. (N) Expression of *Snail* was found in the premigratory neural crest region of wild-type embryos, whereas (P) *Snail* expression was not detected in this region of Sox9$^{-/-}$ embryos.

(Q–X) TUNEL analysis was performed on sections of wild-type and Sox9$^{-/-}$ embryos at E9.5 in the hindlimb region and at E10.5 in the forelimb to hindlimb region. (Q, S, U, and W) At E9.5 and E10.5, wild-type embryos contained few if any apoptotic cells, whereas a significant number of apoptotic cells were observed in premigratory neural crest cells of the caudal (R and X) neural tube and (T and V) migrating trunk neural crest cells in Sox9$^{-/-}$.

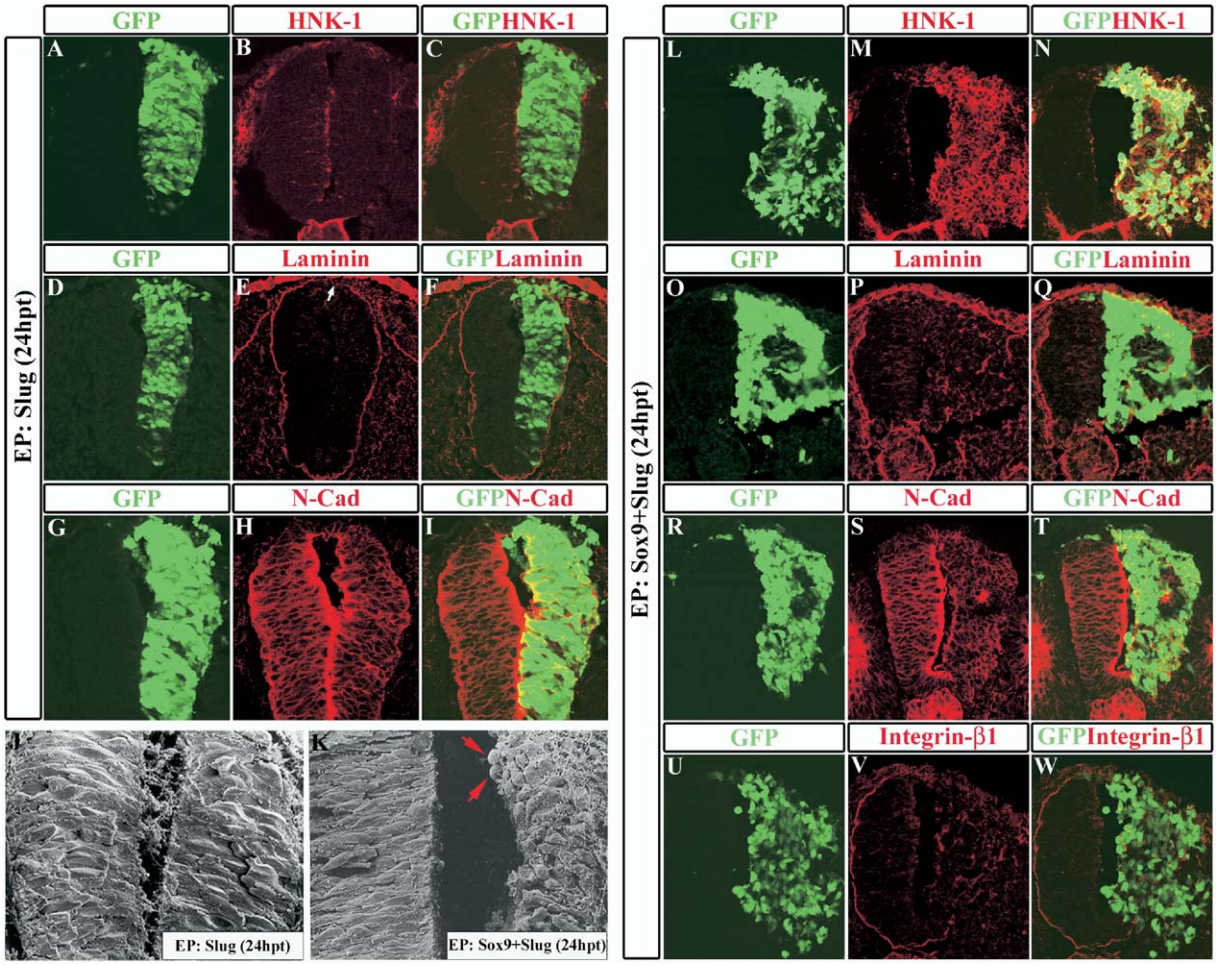

Figure 4. Coexpression of Sox9 and Slug/Snail Induces Neural Crest Delamination and an EMT

(A–J) In ovo electroporation of Slug in HH stage-10 to -11 chick neural tube, assayed 24 hpt. (A–C) Overexpression of Slug does not induce ectopic HNK-1 in the neuroepithelium. (D–F) Most of the transfected cells remain in the neural tube, Laminin expression in the basal membrane remains intact, and a few cells are found delaminating from the dorsal neural tube, where Laminin expression is decreased (arrow). (G–I) Slug transfection does not affect N-Cad expression, and (J) neuroepithelial cells remain in a pseudostratified morphology as shown by electron microscopy.

(L–W) In ovo electroporation of Sox9 and Slug in HH stage-10 to -11 chick neural tubes, assayed 24 hpt. (L–N) Coexpression of Sox9 and Slug induces robust ectopic HNK-1. (O–Q) Most of transfected cells were found migrating out of the neural tube, where Laminin expression was severely disrupted in the basal membrane. (R–T) N-Cad expression continued in transfected cells, but apical polarity was lost. Thus, N-Cad expression was uniform on the cell surface. Electron microscopy indicated that cells in transfected regions of the neural tube were loosely packed, unpolarized, and became more round in appearance (red arrows, [K]). (U–W) Integrin-β1 expression was not induced in Sox9- and Slug-transfected cells but was lost in the basement membrane.

duced in Sox9 mutant embryos compared to the wild-type littermates (Figures 3F and 3H). The expression of *RhoB* followed a similar pattern. *RhoB* was evident in early emigrating NCCs in wild-type and mutant embryos but was absent in mutant embryos from the region in which coalescing DRGs would be expected (Figures 3I–3L). A more dramatic change in the expression of *Snail* expression was detected in the premigratory region of Sox9 null embryos. Wild-type premigratory NCCs express *Snail* (Figures 3M and 3N); however, in Sox9 mutants, *Snail* expression was markedly reduced in the premigratory neural crest region (Figures 3O and 3P). Lack of Sox9 did not affect Snail expression in other regions of the embryo (Figures 3O and 3P). Together, these data suggest that, in the absence of Sox9, NCC induction is initiated; however, expression of *Snail* in premigratory NCCs is absent.

The loss of neural crest derivatives in Sox9 mutant embryos despite the initial induction of NCCs raised the possibility that cells were being eliminated by apoptosis. Consistent with this, we detected a large number of TUNEL-positive cells in the premigratory and early migrating neural crest of E9.5 and E10.5 Sox9 null embryos (Figures 3R–3X). A developmental gradient in the incidence of apoptosis was apparent. In E9.5 embryos and posterior regions of E10.5 embryos, apoptotic cells were largely confined to the premigratory neural crest region (Figures 3R and 3X and Supplemental Figures S3B, S3D, and S3F). In more anterior regions of E10.5 embryos, however, apoptotic cells were evi-

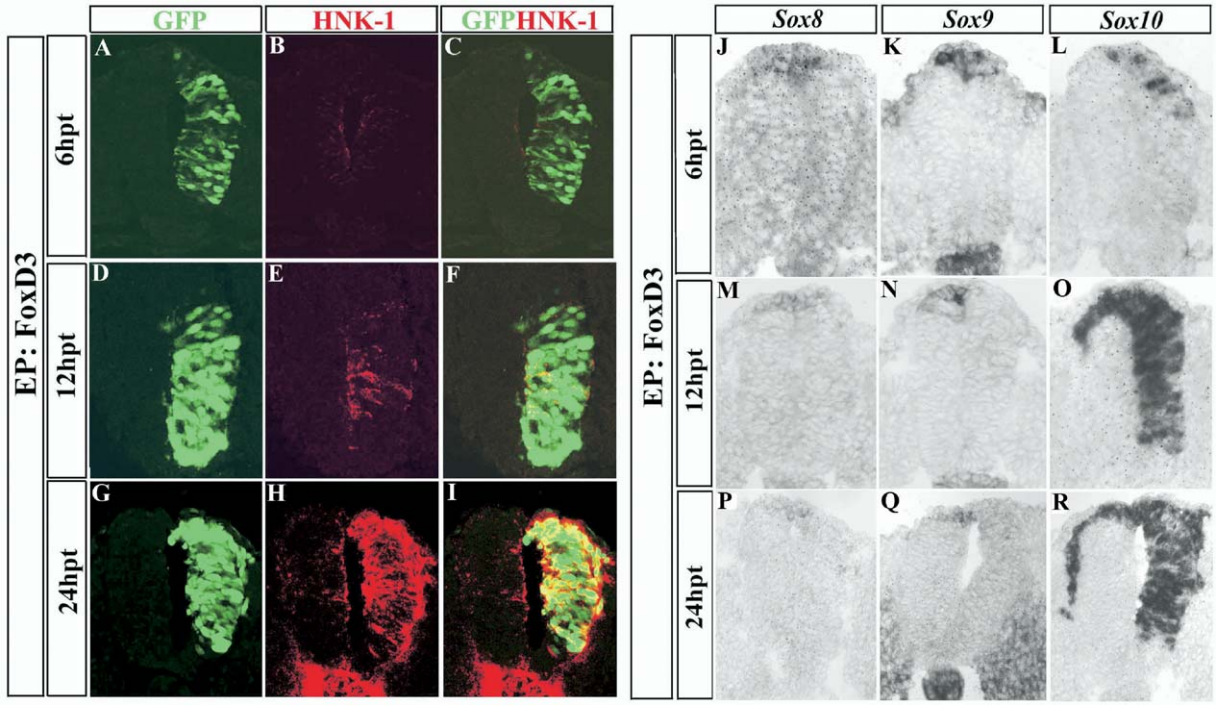

Figure 5. FoxD3 Induces HNK-1 and Sox10 Expression

(A–R) In ovo electroporation of FoxD3 in the chick neural tube at HH stage-10 to -11, analyzed 6 hpt, 12 hpt, and 24 hpt for (B, E, and H) HNK-1 immunofluorescence and (J–R) SoxE genes by in situ hybridization. (A–C) Overexpression of FoxD3 did not induce HNK-1 expression at 6 hpt. (D–F) By 12 hpt, some ectopic HNK-1 expression was detected, and expression became pronounced at (G–I) 24 hpt. Ectopic (J, M, and P) *Sox8* and (K, N, and Q) *Sox9* expression were not detected, but ectopic *Sox10* expression was first seen at (L) 6 hpt and was evident at (O) 12 hpt and (R) 24 hpt.

dent along the migratory route of NCCs, adjacent to the dorsal neural tube (Figures 3T and 3V and Supplemental Figures S3B, S3D, and S3F). Apoptotic cells were rarely observed in wild-type embryos in these positions at equivalent ages and axial levels (Figures 3Q, 3S, 3U, and 3W and Supplemental Figures S3A, S3C, and S3E). These data suggest that, in the absence of Sox9 function, prospective NCCs in the dorsal neural epithelium undergo programmed cell death immediately prior to or shortly after commencing migration into the periphery. This results in marked depletion of neural crest progenitor cells in the periphery and the consequent loss of neural crest derivatives.

Sox9 and Slug/Snail Induce NCC Differentiation and Features of an EMT

The loss of Snail expression in Sox9 null embryos prompted us to address whether Snail was sufficient to mediate some or all of the activities of Sox9. To test this possibility, we used in ovo electroporation in chick embryos. In the chick, the expression profile of Slug and Snail are reversed: Slug is expressed in premigratory NCCs, while Snail expression is found in migratory NCCs (Sefton et al., 1998). Consistent with previous studies (del Barrio and Nieto, 2002), in ovo electroporation of Slug into the trunk neural tube did not induce the expression of ectopic neural crest markers (Figures 4A–4C and data not shown, n = 7); moreover, the transfected neuroepithelial cells remained pseudostratified (Figures 4H and 4J, n = 7) and expression of Laminin, N-Cad, and neural progenitor genes was unaffected (Figures 4D–4I and data not shown, n = 7). Furthermore, the levels of apoptosis in Slug-transfected embryos were similar to those of control embryos (data not shown, n = 5). Electroporation of Snail gave similar results (data not shown). These data suggest that forced expression of Slug/Snail is not sufficient to promote neural crest formation, delamination, or an EMT in trunk neural tube cells.

In contrast, when Sox9 + Slug was coelectroporated, robust ectopic HNK-1 expression was observed (Figures 4L–4N, n = 20) and neural progenitor gene expression was repressed (data not shown). Compared to embryos transfected with Sox9 alone (Figures 1A, 1D, and 1G), in embryos electroporated with Sox9 and Slug, many more transfected cells were found migrating out of the neural tube (Figures 4L, 4O, 4R, and 4U, n = 20). Moreover, the pseudostratified structure of the transfected side of the neural tube appeared to be disorganized, and cells were loosely packed and tended to acquire an unpolarized, rounded morphology (Figures 4K and 4S, n = 20). Consistent with this, the apical concentration of N-Cad expression was lost, although N-Cad expression was maintained on the surface of transfected cells (Figure 4S, n = 20). The activity of Sox9 + Slug appears to be cell autonomous, as individual transfected cells are observed delaminating from the neural tube in situations where the majority of the

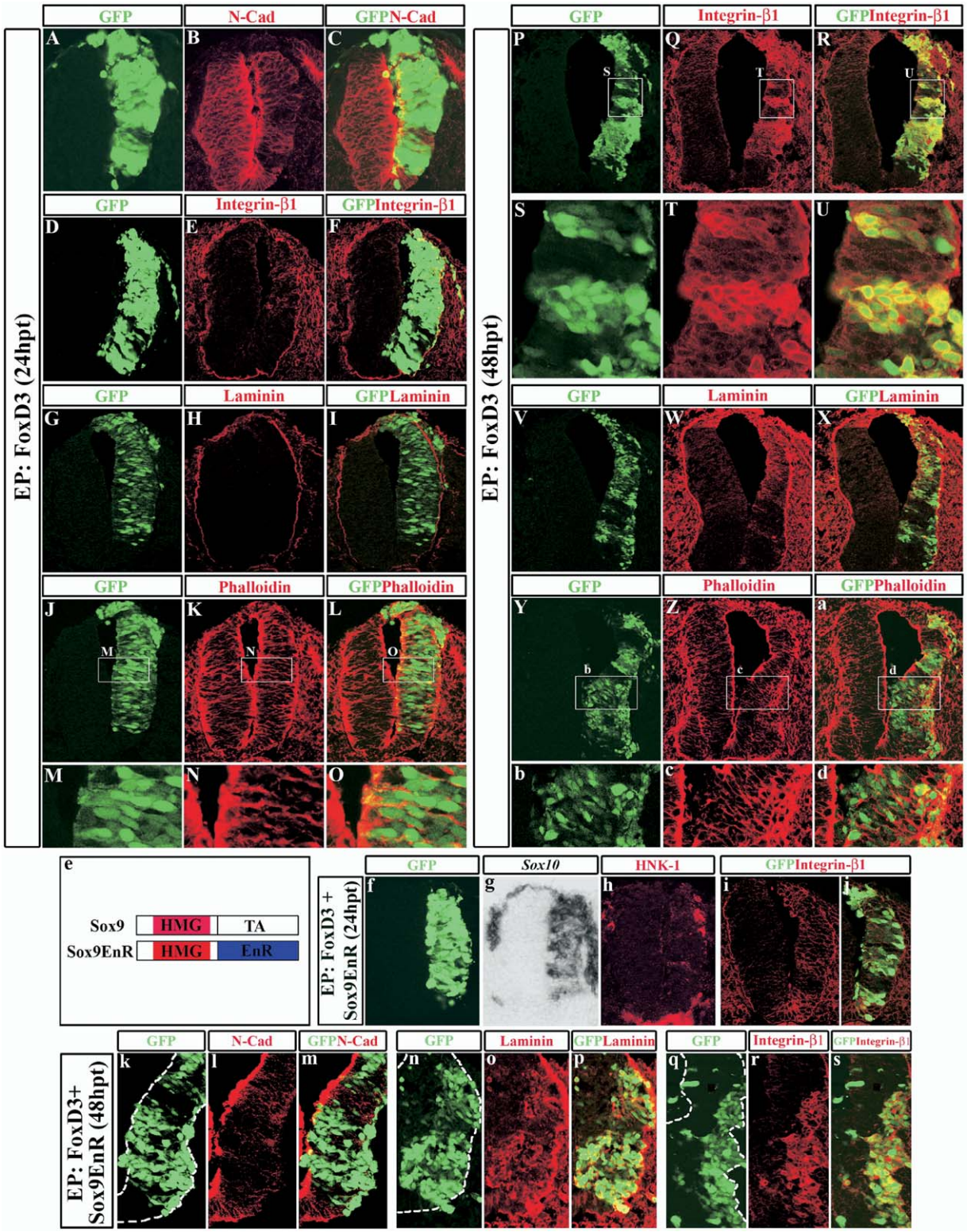

Figure 6. FoxD3 Regulates the Expression of Cell Adhesion Molecules Characteristic of Delaminating Neural Crest Cells Independent of SoxE Gene Function

(A–d) In ovo electroporation of FoxD3 into the chick neural tube at HH stages 10–11, analyzed (A–O) 24 hpt and (P-d) 48 hpt. At 24 hpt, forced expression of FoxD3 begins to (A–C) downregulate N-Cad expression on the surface of neuroepithelial cells and (D–F) induces Integrin-β1 expression. (G–I) In contrast, Laminin expression was not induced in FoxD3-transfected cells at 24 hpt. (J–L) Rhodamine-conjugated phalloidin staining showed that most FoxD3-transfected cells maintained an elongated pseudostratified appearance, as shown in the higher magnification of the region indicated by the (M–O) white box. (P–R) By 48 hpt, pronounced ectopic expression of Integrin-β1 was observed in the

neural tube is untransfected (Supplemental Figures S6J–S6O). Coelectroporation of Sox9 + Snail also gave similar results (data not shown, n = 6). These data suggest that the coexpression of Sox9 and Slug/Snail acts to initiate neural crest-specific gene expression and an EMT.

The similarities between embryos cotransfected with Sox9 + RhoB(QL) and embryos expressing Sox9 + Snail/Slug led us to ask if there was an epistatic connection between RhoB and Snail/Slug. However, no ectopic RhoB expression was observed in cells transfected with Sox9 + Slug (Supplemental Figure S5A, n = 5). Conversely, Sox9 + RhoB(QL) coexpression did not induce ectopic Slug or Snail expression (Supplemental Figures S5B–S5D and data not shown, n = 7). Taken together, these data suggest that Slug/Snail and RhoB do not have a direct epistatic relationship; instead, these proteins may act in parallel or in independent pathways to regulate aspects of an EMT in Sox9-expressing trunk NCCs.

FoxD3 Participates in NCC Induction

The winged-helix transcription factor, FoxD3, has also been implicated in neural crest formation (Dottori et al., 2001; Kos et al., 2001), and FoxD3 expression was maintained in embryos lacking Sox9 (Figures 3A–3D). Forced expression of FoxD3 in chick neural tube cells is sufficient to induce ectopic HNK-1 by 24 hr post-transfection (hpt) (Dottori et al., 2001) (Figures 5G, 5H, and 5I, n = 15). In contrast, the forced expression of Sox9 induced robust ectopic HNK-1 expression within 12 hpt (Cheung and Briscoe, 2003). Moreover, in embryos transfected with FoxD3, ectopic *Sox10* expression was evident by 12 hpt, although ectopic *Sox8* or *Sox9* expression was not detected at any time point examined (Figures 5J–5R). Like members of the SoxE family, the forced expression of FoxD3 was not sufficient to efficiently promote an EMT: the basement membrane was maintained in FoxD3-transfected embryos, and the neural tube retained its tightly packed epithelial configuration (Figures 6G–6I and 6J–6O, n = 10). However, in contrast to SoxE gene expression, FoxD3 inhibited the expression of N-cad and upregulated Integrin-β1 and Laminin expression. Downregulation of N-Cad was evident by 24 hpt (Figures 6A–6C, n = 15), and, at this time, low levels of Integrin-β1 expression could be detected (Figure 6D–6F, n = 12). By 48 hpt, N-Cad expression was no longer detectable (data not shown, n = 18), and high levels of Integrin-β1, Laminin, and Cadherin 7 expression were apparent in FoxD3-expressing cells (Figures 6P–6X and data not shown, n = 15). Labeling with rhodamine-conjugated phalloidin to mark the actin cytoskeleton indicated that most FoxD3-expressing cells retained an elongated, pseudostratified neuroepithelial morphology, although actin bundling and N-Cad expression were lost in some apical regions of the neural tube by 48 hpt (Figures 6Y–6d and data not shown, n = 12). In embryos transfected with Sox9 or Sox9 + Slug, although the distribution of N-Cad is altered in transfected cells, expression of N-Cad continued and Integrin-β1 upregulation was not observed (Figures 1G–1I and 4R–4W, n = 7). These data indicate that, although FoxD3 is not sufficient to induce the morphological features of an EMT, it does appear to regulate the expression of surface proteins characteristic of delaminating NCCs.

To investigate whether the responses to FoxD3 depended on Sox10 induction, we analyzed the effect of blocking SoxE activity. To accomplish this, we constructed a dominant inhibitory SoxE, SoxE^{EnR}, by fusing the HMG domain of Sox9 to the Engrailed transcriptional repressor domain (EnR; Figure 6e). Cotransfection experiments in chick neural tube cells indicated that SoxE^{EnR} is sufficient to inhibit ectopic HNK-1 expression induced by forced expression of Sox9 or Sox10 (data not shown, n = 8). Moreover, electroporation of SoxE^{EnR} into the posterior neural tube of HH stage-10 embryos inhibited induction of endogenous NCCs (data not shown, n = 5). Coelectroporation of SoxE^{EnR} with FoxD3 inhibited HNK-1 induction (Figures 6f and 6h, n = 12) but did not affect the ability of FoxD3 to induce *Sox10* expression (Figure 6g, n = 12). These data suggest that FoxD3 induces *Sox10* expression and that Sox10 initiates HNK-1 expression. In embryos transfected with FoxD3 and SoxE^{EnR}, repression of N-Cad and upregulation of Laminin and Integrin-β1 expression was evident 24 hpt and 48 hpt, and no increase in apoptosis was observed (Figures 6i–6s, n = 6). These data suggest that FoxD3 initiates changes in the cell-cell adhesion properties of NCCs independent of SoxE genes.

Taken together, these data suggest that the combined expression of Sox9, FoxD3, and Slug should be sufficient to specify NCCs and induce an EMT. To test this, we performed in ovo electroporation with a combination of the three genes. In embryos transfected with the three constructs, ectopic HNK-1 expression was observed in GFP$^+$ cells 24 hpt (Figures 7A–7C, n = 9). Phalloidin staining indicated that transfected cells were noticeably more rounded and lacked obvious apical-basal polarity and that the tissue was disorganized and loosely packed (Figures 7D–7F, n = 8). Moreover, in triple-electroporated embryos, transfected cells contained markedly reduced levels of N-Cad expression and had unpregulated Integrin-β1 expression, effects

FoxD3-expressing cells. (S–U) The white box indicated a high magnification of the FoxD3-transfected cells with ectopic Integrin-β1 expression without changing the morphology of the neuroepithelial cells. (V and X) Ectopic Laminin expression began to be observed in FoxD3-transfected cells at 48 hpt. (Y)–(a) Phalloidin staining confirmed that FoxD3-transfected cells remained epithelial-like even though loss of actin bundling occurred in some apical regions of the neural tube (high magnification; [b–d]).

(e–s) (e) Schematic diagram showing full-length Sox9 with the HMG domain and C-terminal transactivation region (TA) and the construction of Sox9^{EnR}, which replaces the C-terminal transactivation region with engrailed repressor domain (EnR). (f) Coelectroporation of FoxD3 and Sox9^{EnR} induces (g) *Sox10* expression, but (h) expression of only a few ectopic HNK-1 cells was detected at 24 hpt. (i) Ectopic expression of Integrin-β1 was observed in FoxD3- and Sox9^{EnR}-transfected cells (j). At 48 hpt, misexpression of FoxD3 and Sox9^{EnR} induced (k–m) repression of N-Cad and upregulation of (n–p) Laminin and (q–s) Integrin-β1 expression.

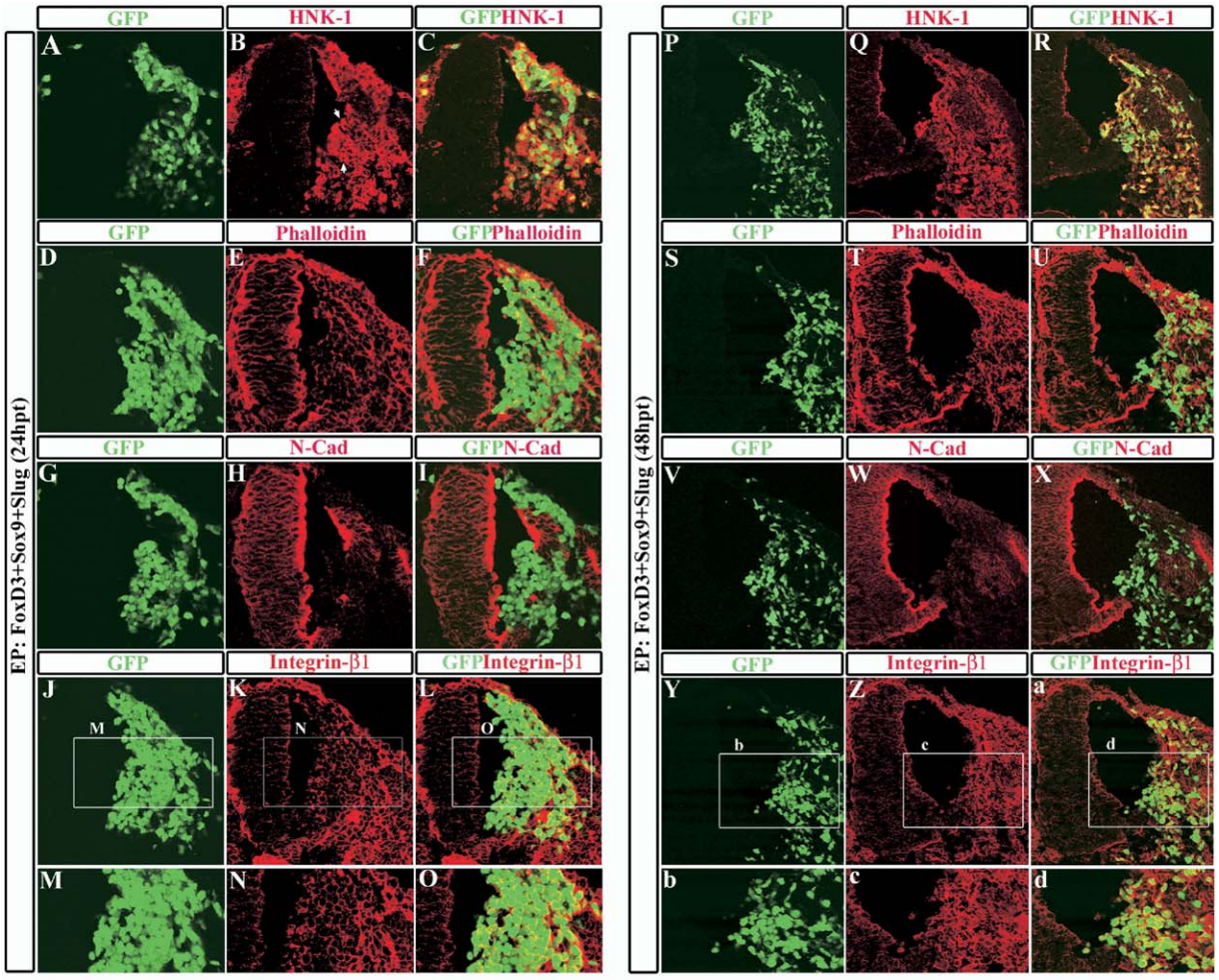

Figure 7. Coexpression of Sox9/FoxD3/Slug Induces Neural Crest Cell Delamination and an EMT and Alteration of Surface Marker Expression

(A–d) The combined in ovo electroporation of Sox9, FoxD3, and Slug into the chick neural tube at HH stages 10–11, analyzed (A–O) 24 hpt and (P-d) 48 hpt. (A–C) Ectopic HNK-1 expression was observed, and transfected cells had lost their pseudostratified epithelia appearance and had taken on the characteristics of rounded mesenchymal-like cells (arrows). (D–F) Rhodamine-conjugated phalloidin staining showed that most triple-transfected cells were round, disorganized, and loosely packed with mesenchymal-like morphology. (G–I) Expression of N-Cad was markedly reduced in transfected cells, and no evidence of polarity was apparent. (J–L) Integrin-β1 expression was induced in some transfected cells, which had a round mesenchymal-like appearance. (M–O) The box shows higher magnification. (A, D, G, and J) Transfected cells were found migrating out of the neural tube from ventral and intermediate regions of the neural tube. (P–R) By 48 hpt, ectopic HNK-1 expression was observed and most of the transfected cells migrated to the mesenchymal and lumen of the neural tube, resulting in depletion of the neuroepithelium and a thinner neural tube, as shown by (S–U) phalloidin staining. (V–X) N-Cad expression was significantly downregulated in the transfected cells. (Y-a) In contrast, Integrin-β1 expression was induced in the transfected cells. (b–d) The box shows higher magnification.

consistent with the forced expression of FoxD3 (Figures 7G–7O, n = 7). Transfected cells were found migrating into surrounding mesenchymal tissue and the lumen of the neural tube (Figures 7A, 7D, 7G, and 7J). By 48 hpt, the effect of triple electroporation was more dramatic, with most transfected cells migrating into mesenchymal tissue. This migration resulted in depletion of neuroepithelium and a thinner neural tube (Figures 7P, 7S, 7V, and 7Y, n = 6). Transfected cells at 48 hpt maintained expression of HNK-1 and Integrin-β1 and downregulation of N-Cad (Figures 7P–7R, 7Y–7d, 7V–7X, n = 5). Phalloidin and Integrin-β1 staining indicated that transfected cells remained round and loosely packed, with mesenchymal-like morphology (Figure 7T, 7Z, and 7c, n = 5). By 72 hpt, transfected cells had migrated extensively within the embryo (Supplemental Figures S6A–S6F). Together, these data indicate that the combined expression of the three cell intrinsic factors are sufficient to coordinately induce the principal features of trunk neural crest cells.

Discussion

A Combinatorial Transcriptional Code for Neural Crest Induction

Neural crest formation involves coordinated changes in gene expression, developmental potential, cell-cell adhesion, and an epithelial-mesenchymal transition. A number of transcription factors are known to be expressed in prospective trunk neural crest cells, including members of the SoxE, Slug/Snail, and FoxD3 families (Spokony et al., 2002; Cheng et al. 2000; Sefton et al. 1998; Dottori et al. 2001; Kos et al. 2001), and we provide evidence that these three families of transcription factors control different aspects of trunk neural crest specification. These data are consistent with those from previous studies that have suggested that the early stages of neural crest development can be divided into distinct steps and that the induction and specification of the neural crest is separable from the initiation of an epithelial-mesenchymal transition (Newgreen and Minichiello 1995; Sela-Donenfield and Kalcheim 1999). Together, these data suggest a genetic network of cell intrinsic factors expressed by trunk NCCs, which regulate and coordinate the induction of various neural crest features (Figure 7e).

Occupying a key position in the network of NCC induction is Sox9. Although cells of the dorsal neural epithelium initiate NCC induction, in the absence of Sox9 they undergo programmed cell death immediately prior to or shortly after commencing migration into the periphery. This correlates with the time at which NCCs undergo an EMT. Consistent with this, apoptosis is induced in neural cells transfected with RhoB, a small G protein implicated in the epithelial-mesenchymal transition of NCCs (Liu and Jessell, 1998), while coexpression of Sox9 and RhoB results in the induction of NCCs and the characteristics of an EMT. Thus, Sox9 appears to protect trunk NCCs undergoing an EMT from apoptosis.

The persistence of FoxD3 expression in embryos lacking Sox9 protein indicates that Sox9 is not required for FoxD3 induction. Conversely, forced expression of FoxD3 does not result in Sox9 induction, suggesting that FoxD3 and Sox9 are induced in parallel in prospective NCCs. Gain-of-function experiments indicate that FoxD3 is able to induce many features of NCC differentiation, including HNK-1 expression, downregulation of the cell adhesion molecule N-Cad, and the upregulation of the mesenchymal marker Integrin-β1 and Laminin. Sox10, a member of the same subgroup of Sox genes as Sox9, in part mediates these activities, as blockade of Sox10 function inhibits FoxD3-mediated HNK-1 induction. In these experiments, however, FoxD3 continues to repress N-Cad and induce Laminin and Integrin-β1. Thus, in addition to inducing Sox10, FoxD3 expression appears to have a distinct role in developing NCCs to control the expression of cell surface proteins that regulate cell adhesive properties. These changes in cell adhesion are not required for cells to undergo an EMT, as forced expression of FoxD3 does not evoke changes in cell behavior typical of an EMT. Conversely, Sox9 + Slug promotes characteristics of an EMT but does not alter the expression of N-Cad or Integrin-β1. Previous studies have provided evidence that the appropriate regulation of cell adhesion molecules in NCCs is required for normal migration (Nakagawa and Takeichi, 1998; Pietri et al., 2004); thus, the regulation of cell adhesion molecules by FoxD3 may be required for targeting NCCs to specific destinations.

Together, the data are most easily accommodated by a model in which the coordinated expression of the three transcription factors is sufficient to initiate an integrated program of neural crest induction that controls the changes in gene expression and cellular properties necessary to specify neural crest cells. What signals regulate these cell intrinsic determinants of neural crest differentiation remains unclear. A number of secreted signaling molecules, notably members of the BMP, FGF, and Wnt families, are expressed in the relevant tissues at appropriate times during development and are implicated in NCC induction (reviewed in Knecht and Bronner-Fraser, 2002). Given the evidence that three cell intrinsic determinants control distinct NCC properties, it is possible that different signals or different combinations of signals induce each of the three genes. Analysis of the molecular basis of the transcriptional control of Sox9, FoxD3, and Slug/Snail may provide insight into this issue.

SoxE Genes Have Multiple and Overlapping Roles in Development

Consistent with the defects in neural crest generation in mouse embryos lacking Sox9, loss-of-function analyses in *Xenopus* indicate that Sox9 is required for neural

(e) Model: Coordinated induction of cell intrinsic factors initiates and integrates the various properties of neural crest cells. FoxD3 regulates the expression of cell-cell adhesion in NCCs by downregulating N-Cad and upregulating Integrin-β1, Laminin, and Cad7 expression. In addition, FoxD3 and Sox9 induce Sox10 expression. SoxE gene expression induces a switch in fate from neural progenitor cells to NCCs and provides competence for NCCs to survive. Coexpression of Sox9 and Slug/Snail induces EMT-like characteristics, altering morphology and apical-basal polarity and dismantling the basement membrane.

crest development in this species too (Spokony et al., 2002). However, neural crest generation in mouse is not completely abrogated in the absence of Sox9. Sox10 represents a candidate compensatory factor that may ameliorate the loss of Sox9. FoxD3 induces Sox10, and expression persists in Sox9 mutant embryos. Moreover, forced expression of Sox10 suggests that it is functionally equivalent to Sox9 (Cheung and Briscoe, 2003). If Sox10 is able to substitute for Sox9, why is there any loss of NCCs in Sox9 mutants? It is possible that the levels of expression of Sox10 are not sufficient to fully compensate for the lack of Sox9. Alternatively, the kinetics of Sox10 induction and the timing of the formation of an EMT may provide an answer. It is possible that, in Sox9 mutants, apoptosis is restricted to those NCCs that initiate an EMT prior to the induction of Sox10 expression. Stochastic variations in the relative timing of these events could therefore account for the differences observed. Furthermore, it is possible that the relationship between the induction of Sox10 and the initiation of an EMT differs along the anterior-posterior axis and between species, resulting in the observed variations in the requirement for Sox9 and Sox10. Consistent with this idea, while loss of Sox10 has no effect on the early development of NCCs in mouse (Kim et al., 2003), loss-of-function experiments in *Xenopus* provide evidence for a requirement for Sox10 in NCC induction in this species (Honorè et al., 2003).

In addition to playing roles in the early specification of NCCs, SoxE genes have also been implicated in later aspects of neural crest development and in the development of other tissues. Sox10 appears to maintain the multipotency and inhibit neuronal differentiation of NCCs and is required for the differentiation of a number of neural crest derivatives (Kim et al., 2003; Southard-Smith et al., 1998; Britsch et al., 2001). The role of Sox9 in the later generation of neural crest derivatives has been more difficult to assess because, although residual PNS neurons and glial are generated, Sox9-deficient embryos die at ~e11.5, precluding a more detailed analysis. Removal of Sox9 from the neural crest by using a Wnt1-Cre, which deletes Sox9 at a later developmental time point and in a more restricted population of cells than the strategy used in this study, results in a complete absence of cartilage and endochondral bones derived from cranial neural crest cells (Mori-Akiyama et al., 2003). This is consistent with the previously identified role for Sox9 in mesenchymal condensation and the generation of skeletal structures (Bi et al., 1999; Akiyama et al., 2002). The multiple roles assigned to the SoxE genes suggest that these transcription factors function in a context-dependent manner at least in part by interacting with different cofactors (Kamachi et al., 2000). Whether the neural crest function of SoxE genes requires a partner(s) remains to be determined, as does the identity of any putative cofactor.

Cell Competence Regulates Epithelial-Mesenchymal Transitions

Previous studies of neural crest development have suggested roles for both the Slug/Snail family of transcription factors and the small G protein, RhoB, in the formation of a neural crest EMT and delamination (Nieto et al., 1994; Liu and Jessell, 1998). Our studies suggest that SoxE transcription factors are also key players in this transformation. In the absence of SoxE expression, Slug/Snail is not sufficient to induce an EMT in trunk neural cells. In contrast, expression of RhoB in neural tube cells lacking SoxE gene expression results in the apoptotic elimination of cells, suggesting that the inappropriate initiation of an EMT is not compatible with the survival of neural epithelial cells. Together, these data indicate that SoxE gene expression provide the appropriate competence to neural cells so that an EMT can be completed successfully.

Which cell intrinsic factors are normally involved in the EMT of NCCs remains to be clarified. Mouse embryos lacking Snail do not survive beyond midgestation, precluding the analysis of the role of Snail in mammalian neural crest generation (Carver et al., 2001). In chick, RNA antisense experiments have provided evidence supporting a role for Slug in NCC development (Nieto et al., 1994), but a number of observations have suggested that Slug expression does not necessarily correlate with the EMT (Sela-Donenfield and Kalcheim, 1999). Slug/Snail has, however, been shown to confer resistance to cell death in neural crest cells (Vega et al., 2004). It is possible, therefore, that the loss of Snail expression could be one of the factors that facilitates the apoptotic elimination of neural crest progenitors in Sox9 mutants. Whether Rho activity is essential for an EMT during normal neural crest development is also unclear, as although a blockade of Rho activity inhibits neural crest delamination in chick (Liu and Jessell, 1998), neural crest development proceeds normally in mice harboring a null allele of RhoB (Liu et al., 2001). These findings may be reconciled by the presence, in NCCs, of other small G proteins that compensate for the loss of RhoB.

The delamination and EMT of NCCs involves changes in cell shape and cell-cell interaction, loss of apicobasal polarity, and disassembly of the basement membrane. These are characteristics common to metastatic transformation and acquisition of invasive behavior by tumor cells. Previously, expression of Slug/Snail in tumors has been associated with increased invasiveness, and the induction of Slug/Snail in cell line models has been demonstrated to enhance their metastatic characteristics (Cano et al., 2000; Batlle et al., 2000). Our findings would suggest, however, that not all epithelial cells are competent to undergo an EMT; moreover, in some circumstances, compelling cells to initiate an EMT appears to be incompatible with their continued survival. The expression of SoxE in neural cells is sufficient to confer competence to successfully complete an EMT, and it will be interesting to determine if SoxE proteins or other competence/survival factors are important for the transformation of epithelial tumors into invasive carcinomas. Whether this is the case or not, the data presented here contribute to our understanding of the cell intrinsic network that regulates early neural crest development and may provide insight into other cases in which an EMT and transcriptional programs are coordinately regulated.

Experimental Procedures

Mouse Mutant Lines

Introduction of LoxP sites into the Sox9 gene and the generation of Sox9 Floxed heterozygous and homozygous mice were described

previously (Akiyama et al., 2002). Generation of Sox9 null embryos by using Cre transgenes driven by germ line-specific promoters was performed as described (Akiyama et al., 2004).

In Situ Hybridization, Immunohistochemistry, and Tunel Assays

In situ hybridization was performed as described (Cheng et al., 2000). The following probes were used: chick Sox8, Sox9, and Sox10 (Cheng et al., 2000, 2001); rat Isl-1/2 (T. Jessell); mouse Sox10 (M. Wegner), Wnt1, Wnt6 (A. McMahon), GDF7 (A. Simeone), ErbB3 (J. Golding), Brn3.0 (S-L. Ang), Notch-1 (M. Logan), and RhoB (T. Jessell); and a Lmx1a chick-expressed sequence tag (EST) clone. FoxD3 (I.M.A.G.E. ID 418507) and Snail (I.M.A.G.E. ID 5121591) probes were derived from I.M.A.G.E Consortium cDNA Clones. Immunohistochemical detection of proteins on sections was performed as described (Briscoe et al., 2000). Antibodies against the following proteins were used: green fluorescence protein (Molecular Probes); HNK-1 (Becton Dickinson); Laminin (Sigma); N-Cad (Zymed Labs); β-tubulin (Covance); Zic2 (Brown et al., 2003); Sox9 (M. Wegner); Phox2b (J. Brunet); Mash1 (F. Guillemot); Math1 (J. Johnson); Nkx6.1 (H. Edlund); Olig2 (B. Novitch); Isl-1/2 (Ericson et al., 1992); Pax7, Pax6, HB9, Nkx2.2, Shh, and Chx10 (Briscoe et al., 2000); Pax3 (Baker et al., 2002); Integrin-β (Hayachi et al., 1990); Msx1/2 (Liem et al., 1995); Lim1/2; LH2A/B (Lee et al., 1998); and Rhodamine Phalloidin (Molecular Probes). Antisera against Sox10 were generated in rabbit against the peptide CPPAHSPTANWDQPVYTTLT. Apoptosis analysis on frozen sections was carried out by using the in situ cell death detection kit (Roche), and whole-mount TUNEL analysis was performed by using the in situ apoptosis detection kit based on the manufacturer's instructions (Qbiogene).

Chick In Ovo Electroporation

Fertilized chick eggs were obtained from Winter Egg Farm (Royston, UK). Embryos were staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951). In ovo electroporation was carried out as described (Cheung and Briscoe, 2003). The following constructs were used: chick Sox9 cDNA (Kamachi et al., 1999), chick Sox10 cDNA (Cheng et al., 2000), $SoxE^{EnR}$ (transactivation domain of chick Sox9 containing amino acid 265–495 replaced by Engrailed transcriptional repressor domain; Kamachi et al., 1999), chick Slug cDNA (del Barrio and Nieto, 2002), and mouse FoxD3 cDNA (P.A. Labosky). These constructs were inserted upstream of an internal ribosomal entry site (IRES) and a nuclear localization sequence (nls)-tagged GFP in the pCAGGS expression vector (Niwa et al., 1991). Wild-type RhoB, constitutive active (QL), and inactive forms (TN) of RhoB were in the pcDNA3 vector (Invitrogen) provided by H. Mellor (Mellor et al., 1998).

Supplemental Data

Supplemental Data including Supplemental Figures S1–S6 are available at http://www.developmentalcell.com/cgi/content/full/8/2/179/DC1/.

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References

Akitaya, T., and Bronner-Fraser, M. (1992). Expression of cell adhesion molecules during initiated and cessation of neural crest migration. Dev. Dyn. *194*, 12–20.

Akiyama, H., Chaboissier, M.-C., Martin, J.F., Schedl, A., and de Crombrugghe, B. (2002). The transcription factor Sox9 has essential roles in successive steps of the chrondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes Dev. *16*, 2813–2828.

Akiyama, H., Chaboissier, M.-C., Behringer, R.R., Rowitch, D.H., Schedl, A., Epstein, J.A., and de Crombrugghe, B. (2004). Essential role of Sox9 in the pathway that controls formation of cardiac valves and septa. Proc. Natl. Acad. Sci. USA *101*, 6502–6507.

Baker, C.H.V., Stark, M.R., and Bronner-Fraser, M. (2002). Pax3-expressing trigeminal placode cells can localize to trunk neural crest sites but are committed to a cutaneous sensory neuron fate. Dev. Biol. *249*, 219–236.

Batlle, E., Sancho, E., Franci, C., Dominguez, D., Monfar, M., Baulida, J., and Garcia De Herreros, A. (2000). The transcription factor Snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat. Cell Biol. *2*, 84–89.

Bi, W., Deng, J.M., Zhang, Z., Behringer, R.R., and de Crombrugghe, B. (1999). Sox9 is required for cartilage formation. Nat. Genet. *22*, 85–89.

Bi, W., Huang, W., Whitworth, D.J., Deng, J.M., Zhang, Z., Behringer, R.R., and de Crombrugghe, B. (2001). Haploinsufficiency of Sox9 results in defective cartilage primordial and premature skeletal mineralization. Proc. Natl. Acad. Sci. USA *98*, 6698–6703.

Bolos, V., Peinado, H., Perez-Moreno, M.A., Fraga, M.F., Esteller, M., and Cano, A. (2003). The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors. J. Cell Sci. *116*, 499–511.

Briscoe, J., Alessandra, P., Jessell, T.M., and Ericson, J. (2000). A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. Cell *101*, 435–445.

Britsch, S., Goerich, D.E., Riethmacher, D., Peirano, R.I., Rossner, M., Nave, K.A., Birchmeier, C., and Wegner, M. (2001). The transcription factor Sox10 is a key regulator of peripheral glial development. Genes Dev. *15*, 66–78.

Brown, L.Y., Kottmann, A.H., and Brown, S. (2003). Immunolocalization of Zic2 expression in the developing mouse forebrain. Gene Expr. Paterns *3*, 361–367.

Cano, A., Pérez, M.A., Rodrigo, I., Locascio, A., Blanco, M.J., Del Barrio, M.G., Portillo, F., and Nieto, M.A. (2000). The transcription factor Snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat. Cell Biol. *2*, 76–83.

Carver, E.A., Jiang, R., Lan, Y., Oram, K.F., and Gridley, T. (2001). The mouse Snail gene encodes a key regulator of the epithelial-mesenchymal transition. Mol. Cell. Biol. *21*, 8184–8188.

Cheng, Y.-C., Cheung, M., Abu-Elmagd, M.M., Orme, A., and Scotting, P.J. (2000). Chick Sox10, a transcription factor expressed in both early neural crest cells and central nervous system. Brain Res. Dev. Brain Res. *121*, 233–241.

Cheng, Y.-C., Lee, C.J., Badge, R.M., Orme, A.T., and Scotting, P.J. (2001). Sox8 gene expression identifies immature glial cells in developing cerebellum and cerebellar tumours. Brain Res. Mol. Brain Res. *92*, 193–200.

Cheung, M., and Briscoe, J. (2003). Neural crest development is regulated by the transcription factor Sox9. Development *130*, 5681–5693.

del Barrio, M.G., and Nieto, M.A. (2002). Overexpression of Snail family members highlights their ability to promote chick neural crest formation. Development *129*, 1583–1593.

Dottori, M., Gross, M.K., Labosky, P., and Goulding, M. (2001). The

winged-helix transcription factor Foxd3 suppresses interneuron differentiation and promotes neural crest cell fate. Development *128*, 4127–4138.

Duband, J.L., Monier, F., Delannet, M., and Newgreen, D. (1995). Epithelium-mesenchyme transition during neural crest development. Acta Anat. *154*, 63–78.

Ericson, J., Thor, S., Edlund, T., Jessell, T.M., and Yamada, T. (1992). Early stages of motor neuron differentiation revealed by expression of homeobox gene Islet-1. Science *256*, 1555–1560.

Guillemot, F., Lo, L., Johnson, J.E., Auerbach, A., Anderson, D.J., and Joyner, A.L. (1993). Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell *75*, 463–476.

Hamburger, H., and Hamilton, H.L. (1951). A series of normal stages in the development of the chick embryo. J. Morphol. *88*, 49–92.

Hayachi, Y., Haimovich, B., Reszka, A., Boettinger, D., and Horwitz, A. (1990). Expression and function of chicken integrin-β1 subunit and its cytoplasmic domain mutants in mouse NIH 3T3 cells. J. Cell Biol. *110*, 175–184.

Honorè, S., Aybar, M., and Mayor, R. (2003). Sox10 is required for the early development of the prospective neural crest in *Xenopus* embryos. Dev. Biol. *260*, 79–96.

Kamachi, Y., Cheah, K.S.E., and Kondoh, H. (1999). Mechanism of regulatory target selection by the SOX high-mobility-group domain proteins as revealed by comparison of SOX1/2/3 and SOX9. Mol. Cell. Biol. *19*, 107–120.

Kamachi, Y., Uchikawa, M., and Kondoh, H. (2000). Pairing SOX off with partners in the regulation of embryonic development. Trends Genet. *16*, 182–187.

Kim, J., Lo, L., Dormand, E., and Anderson, D.J. (2003). SOX10 maintains multipotency and inhibits neuronal differentiation of neural crest stem cells. Neuron *38*, 17–31.

Knecht, A.K., and Bronner-Fraser, M. (2002). Induction of the neural crest: a multigene process. Nat. Rev. Genet. *3*, 453–461.

Kos, R., Reedy, M.V., Johnson, R.L., and Erickson, C.A. (2001). The winged-helix transcription factor FoxD3 is important for establishing the neural crest lineage and repressing melanogenesis in avian embryos. Development *128*, 1467–1479.

LaBonne, C., and Bronner-Fraser, M. (2000). Snail-related transcriptional repressors are required in *Xenopus* for both the induction of the neural crest and its subsequent migration. Dev. Biol. *221*, 195–205.

Le Douarin, N.M., and Kalcheim, C. (1999). The Neural Crest (Cambridge, UK: Cambridge University Press).

Lee, K.J., Mendelsohn, M., and Jessell, T.M. (1998). Neuronal patterning by BMPs: a requirement for GDF7 in the generation of a discrete class of commissural interneurons in the spinal cord. Genes Dev. *12*, 3394–3407.

Liem, K., Tremml, G., Roelink, H., and Jessell, T.M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. Cell *82*, 969–979.

Liu, J., and Jessell, T.M. (1998). A role for RhoB in the delamination of neural crest cells from the dorsal neural tube. Development *125*, 5055–5067.

Liu, A.-X., Rane, N., Liu, J.-P., and Prendergast, G.C. (2001). RhoB is dispensable for mouse development, but it modifies susceptibility to tumor formation as well as cell adhesion and growth factor signalling in transformed cells. Mol. Cell. Biol. *21*, 6906–6912.

Mellor, H., Flynn, P., Nobes, C.D., Hall, A., and Parker, P.J. (1998). PRK1 is targeted to endosomes by the small GTPase, RhoB. J. Biol. Chem. *273*, 4811–4814.

Mori-Akiyama, Y., Akiyama, H., Rowitch, D.H., and de Crombrugghe, B. (2003). Sox9 is required for determination of the chondrogenic cell lineage in the cranial neural crest. Proc. Natl. Acad. Sci. USA *100*, 9360–9365.

Nakagawa, S., and Takeichi, M. (1998). Neural crest emigration from the neural tube depends on regulated cadherin expression. Development *125*, 2963–2971.

Newgreen, D.F., and Minichiello, J. (1995). Control of epitheliomesenchymal transformation. I. Events in the onset of neural crest cell migration are separable and inducible by protein kinase inhibitors. Dev. Biol. *170*, 91–101.

Nieto, M.A., Sargent, M.G., Wilkinson, D.G., and Cooke, J. (1994). Control of cell behavior during vertebrate development by slug, a zinc finger gene. Science *264*, 835–839.

Niwa, H., Yamamura, K., and Miyazaki, J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene *15*, 193–199.

Pattyn, A., Morin, X., Cremer, H., Goridis, C., and Brunet, J. (1999). The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. Nature *399*, 366–370.

Pietri, T., Eder, O., Breau, M.A., Topilko, P., Blanche, M., Brakebusch, C., Fässler, R., Thiery, J.P., and Dufour, S. (2004). Conditional β1-integrin gene deletion in neural crest cells causes severe developmental alternations of the peripheral nervous system. Development *131*, 3871–3883.

Savagner, P. (2001). Leaving the neighborhood: molecular mechanisms during epithelial-mesenchymal transition. Bioessays *23*, 912–923.

Sefton, M., Sanchez, S., and Nieto, M.A. (1998). Conserved and divergent roles for members of the Snail family of transcription factors in the chick and mouse embryo. Development *125*, 3111–3121.

Sela-Donenfield, D., and Kalcheim, C. (1999). Regulation of the onset of neural crest migration by coordinated activity of BMP4 and Noggin in the dorsal neural tube. Development *126*, 4749–4762.

Serbedzija, G.N., Fraser, S.E., and Bronner-Fraser, M. (1990). Pathways of trunk neural crest migration in the mouse embryo as revealed by vital dye labelling. Development *108*, 605–612.

Southard-Smith, E.M., Kos, L., and Pavan, W.J. (1998). Sox10 mutation disrupts neural crest development in Dom Hirschsprung mouse model. Nat. Genet. *18*, 60–64.

Spokony, R.F., Aoki, Y., Saint-Germain, N., Magner-Fink, E., and Saint-Jeannet, J.-P. (2002). The transcription factor Sox9 is required for cranial neural crest development. Development *129*, 421–432.

Vega, S., Morales, A.V., Ocaña, O.H., Valdés, F., Fabregat, I., and Nieto, M.A. (2004). Snail blocks the cell cycle and confers resistance to cell death. Genes Dev. *18*, 1131–1143.